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RESEARCH ARTICLE

Development of an immunological technique for identifying multiple predator–prey interactions in a complex arthropod assemblage

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Circadian feeding; ELISA; inclusion/exclusion cages; intraguild predation; predator assemblage; predator gut content analysis; prey marking.

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Abstract

A simplified but highly effective approach for the post-mortem evaluation of predation on several targeted members of an arthropod assemblage that does not require the development of pest-specific enzyme-linked immunosorbent assay (ELISA) (e.g. pest-specific monoclonal antibodies) or PCR assays (DNA primers) is described. Laboratory feeding studies were conducted to determine if predation events could be detected from predators that consumed prey marked with foreign protein. I determined that large and small rabbit immunoglobulin G (IgG)-marked prey can be detected by a rabbit-IgG-specific ELISA in the guts of chewing and piercing–sucking type predators. I then conducted multifaceted inclusion and exclusion field cage studies to qualify the degree of interguild and intraguild predation occurring among a complex arthropod assemblage during four separate light phase treatments. The field cages contained an arthropod assemblage consisting of 11 or 12 species of predaceous arthropods and three pest species. The three pests introduced into the cages included third instar *Trichoplusia ni* marked with rabbit IgG, third instar *Lygus hesperus* marked with chicken IgG and *Pectinophora gossypiella* sentinel egg masses. The inclusion cages allowed foraging fire ants, *Solenopis xyloni*, to freely enter the cages while the exclusion cages contained barriers that prevented ant entry. The results obtained using the conventional inclusion/exclusion field cage methodology revealed that there was substantial interguild and intraguild predation occurring on the majority of the arthropods in the assemblage, particularly in those cages that included ants. I then precisely identified which predators in the assemblage were feeding on the three targeted pests by conducting three post-mortem gut content analyses on each individual predator (1503 individuals) in the assemblage. Specifically, *P. gossypiella* egg predation events were detected using an established *P. gossypiella*-egg-specific ELISA, and third instar *T. ni* and *L. hesperus* predation events were detected using rabbit-IgG-specific and chicken-IgG-specific ELISAs, respectively. Generally, the gut ELISAs revealed that *Collops vittatus*, *Spanagonicus albofasciatus* and *Geocoris punctipes* readily preyed on *P. gossypiella* eggs; *Nabis alternatus*, *Zelus renardii* and spiders (primarily *Misumenops celer*) readily preyed on marked *L. hesperus* nymphs, and spiders, *S. albofasciatus* and *N. alternatus* readily preyed on *T. ni* larvae. Furthermore, the cage methods and the post-mortem predator gut ELISAs revealed very few distinctive patterns of predation with regard to the light cycle the assemblage was exposed to.

Introduction

Predaceous arthropods are important regulators of arthropod populations (Symondson *et al.*, 2002); however, identifying the feeding choices and amount of prey consumed by any given generalist predator community is very difficult. Direct visual field observations of predation are tedious, time consuming and extraordinarily difficult to obtain because most arthropods are small, elusive, cryptic (Hagler *et al.*, 1991) and may feed at night (Pfannenstiel & Yeargan, 2002). Moreover, most predators do not leave evidence of attack. Perhaps, the most frequently used experimental approach for evaluating arthropod predation in nature is through studies conducted in field cages (Luck *et al.*, 1988). Such studies require manipulation of either the predator(s) or pest(s) population within the cage by either removing or introducing certain members of the assemblage. Mortality is then estimated based on absence of the insects in the assemblage over time (Smith & De Bach, 1942). Such studies have documented the qualitative impact of manipulated predator assemblages on many types of pests and natural enemies, but they do not provide direct evidence of which predators in the assemblage are exerting the greatest biological control. Often, the only conclusive evidence of arthropod predation can be found in the stomach contents of predators. Unfortunately, direct visual or microscopic analyses of the stomach contents of most predators is impossible because chewing type predators chew their prey into tiny, unrecognisable pieces and piercing–sucking predators liquefy prey contents (Cohen, 1995). Indirect techniques of stomach analysis, including the use of radioactive markers (Baldwin *et al.*, 1955; Nuessly & Sterling, 1986) or rare elements (Shepard & Waddill, 1976; Johnson & Reeves, 1995) for tagging potential prey and electrophoresis (Murray & Solomon, 1978; Giller, 1982) for identification of prey-specific proteins have been used to identify prey, but these techniques pose potential dangers to users and the environment (e.g. radioactive markers), are too time consuming or do not possess the necessary specificity and sensitivity. Currently, the state-of-the-art predator stomach content assays include enzyme-linked immunosorbent assays (ELISA) for the detection of pest-specific proteins (Greenstone, 1996) and PCR assays for the detection of pest-specific DNA (Agustí *et al.*, 2003; Harper *et al.*, 2005). However, only a few researchers have exploited the precision of molecular gut content assays for measuring arthropod predation in the field because the development of such assays is costly, time consuming and requires technical expertise. Furthermore, many of these field studies (especially DNA-based techniques) use very small data sets, making ecological or biological inferences difficult. Consequently, post-mortem gut content assays are not well suited for studying predation on a multitude of prey species (see Sheppard & Harwood, 2005, for a review).

Due to the limitations of the current methods used for studying arthropod predation, the overwhelming majority of studies have focused solely on the consumption of economically important pests (Ives *et al.*, 2005). Moreover, as Lang (2003) accurately points out, most field studies of predation only examine one predator or one pest species at a time. In short, relatively little is known about the food web dynamics of predators in complex arthropod assemblages.

The many shortcomings of the current methods used to assess predation were the impetus for me to develop a simplified immunological technique that can be used to analyse predator gut contents for many types of prey. The first part of this study is a 'feasibility' study that describes how to mark large and small insect prey with foreign protein [rabbit immunoglobulin G (IgG)] and how to analyse the gut contents of predators that consumed the protein-marked prey by a protein-specific ELISA. The second part of the study assesses the degree of interguild and intraguild predation occurring in an arthropod assemblage inhabiting cotton using conventional exclusion and inclusion field cage methodology. The field cages had an assemblage of arthropods typically found in cotton. The exclusion cages prevented foraging fire ants, *Solenopsis xyloni* McCook, from entering the cages while the inclusion cages allowed the fire ants to move freely into and out of the cages. The percent mortality of each member of the assemblage (pest and predator) was assessed over four different 6-h light cycle treatments. The final part of my study consisted of analysing the gut contents of every predator in the field cages for the presence of three targeted arthropods in the assemblage. Specifically, I analysed the gut contents of 1503 individual predators for the presence of pink bollworm, *Pectinophora gossypiella* (Saunders) egg antigen, rabbit-IgG-marked cabbage looper, *Trichoplusia ni* (Hübner) larvae and chicken-IgG-marked western tarnished plant bug, *Lygus hesperus* Knight nymph remains. In this article, I describe how the methodology can be expanded and applied to a wide variety of predator studies and I discuss the advantages and limitations of the methodology.

Materials and methods

Laboratory tests

Laboratory tests were conducted to determine if protein-marked prey could be detected in predator guts at various time intervals after they eat a single marked prey item. The

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predator and prey insects included the striped earwig, *Labidura riparia* (Pallas), a large (20 mm) chewing predator that fed on a large (15 mm) protein-marked pink bollworm larva and the minute pirate bug, *Orius tristicolor* (White), a small (2 mm) piercing and sucking predator that fed on a very small (<1 mm) protein-marked sweetpotato whitefly, *Bemisia tabaci* (Gennadius) Biotype B (=*Bemisia argentifolii* Bellows & Perring) parasitoid, *Eretmocerus* sp.

Earwig/pink bollworm feeding trial

Pink bollworm larvae were marked using the method of Hagler & Miller (2002). Technical grade rabbit IgG (Sigma Chemical Co., St Louis, MO, USA, No. I-8140) was mixed into pink bollworm artificial diet (Adkisson *et al.*, 1960) just prior to the solidification of the diet. The final concentration of the rabbit-IgG-enriched diet was 1.13 mg of rabbit IgG per millilitre of diet. Fourth instar pink bollworm larvae were removed from their regular diet and placed on the rabbit-IgG-enriched diet for 24 h. After 24 h, a single larva was removed from the enriched diet and placed in a 5.5-cm-diameter Petri dish containing an adult earwig. Immediately after an earwig was observed consuming an entire marked pink bollworm larva, it was removed from the dish and placed into a clean Petri dish containing a wetted sponge (to prevent desiccation) and held for 0, 24 or 48 h at 27°C and then frozen at −70°C. Individual earwigs were then homogenised in 500 μL of tris-buffered saline (TBS) (pH 7.4) and assayed for the presence of pink bollworm remains (rabbit IgG) by the anti-rabbit IgG-specific sandwich ELISA described below.

Minute pirate bug/*Eretmocerus* feeding trial

Adult *Eretmocerus* sp. [Ethiopia, M96076 (the *Eretmocerus* sp. used were originally collected in Ethiopia by G. Terefe and D. Gerling and screened by J. Goolsby at the USDA-APHIS, Mission Biological Control Center, Mission, TX, USA. The #M96076 indicates the 76th accession of 1996 for the Mission Biological Control Center quarantine)] were internally marked using the method of Hagler *et al.* (2002). As the parasitoids emerged from their host (immature *B. tabaci*), they were placed in a 2.5-L Tupperware® container and held at 27°C. The container's lid had a 6.0-cm-diameter hole covered with muslin (organdy) fabric to facilitate air exchange. The parasitoids were provided honey for food, which contained 5.0 mg of rabbit IgG per millilitre of honey. The parasitoids were presented with the honey by dipping a toothpick into the honey and streaking several thin lines of the honey (≈25 μL) on the underside of the Tupperware lid. The parasitoids were allowed to feed freely on the diet for 24 h. The parasitoids were then externally marked with rabbit IgG using a medical nebuliser (Sunrise Medical, Somerset, PA, USA; model #800D) as described by Hagler & Jackson (1998). Briefly, 2.0 mL of a water solution containing 10 mg of rabbit IgG was placed into the nebuliser. The hose of the nebuliser was inserted into a 2.5-cm hole (just slightly larger than the mouth of the nebuliser) that was punched out of the side of the Tupperware container. The air outlet was turned on, and the parasitoids were 'fogged' until there was no more rabbit IgG solution remaining in the nebuliser (≈2.0 min). The nebuliser was removed from the container, and the 2.5-cm hole in the Tupperware was plugged with a cork. The parasitoids were held in the container for 1 h after fogging and then placed into a Petri dish containing a single adult minute pirate bug. Immediately after the minute pirate bug ate a single marked parasitoid, it was removed from the dish and placed into a clean Petri dish containing a wetted sponge (to prevent desiccation) and held for 0, 3, 6, 9 or 12 h at 27°C and then frozen at −70°C. Individual minute pirate bugs were then homogenised in 500 μL of TBS and assayed for the presence of parasitoid remains (rabbit IgG) by the anti-rabbit IgG-specific ELISA described below.

Anti-rabbit IgG sandwich ELISA

A double antibody anti-rabbit IgG sandwich ELISA was performed on every individual predator (Hagler *et al.*, 1992*a*). Each well of a 96-well ELISA microplate was coated with 100 μL of anti-rabbit IgG (developed in goat) (Sigma Chemical Co., No. R-2004) diluted 1:500 in double distilled (dd) H_2O and incubated overnight at 4°C. The anti-rabbit IgG was discarded and 360 μL of 1% nonfat dry milk in ddH_2O was added to each well for 30 min at 27°C to block any remaining nonspecific binding sites on the plates. After the nonfat milk was removed, a 100 μL aliquot of a homogenised predator sample was placed in an individual well of the pretreated assay plate and incubated for 1 h at 27°C. Predator samples were then discarded and each well was briefly rinsed three times with TBS Tween 20 (0.05%) and twice with TBS. Aliquots (50 μL) of anti-rabbit IgG conjugated to horseradish peroxidase (Sigma Chemical Co., No. A-6154) diluted to 1:1000 in 1.0% nonfat dry milk in ddH_2O were added to each well for 1 h at 27°C. Plates were again washed as described above and 50 μL of substrate was added using the reagents supplied in a horseradish peroxidase substrate kit (Bio-Rad, Richmond, CA, USA, No. 172-1064). Following substrate incubation (2 h), the optical absorbance of each well was measured with a microplate reader set at 405 nm.

The mean [±standard deviation (SD)] ELISA absorbance value and the percentage of predators scoring positive for IgG remains were tallied for each postmeal-holding interval.

Negative predator controls

Predators serving as negative controls (n = 8 per ELISA plate) were collected from cotton and alfalfa fields located at the University of Arizona's Agricultural Research Station, Maricopa, AZ, USA. Individual predators were placed in 500 μL TBS, macerated and frozen (−70°C) until assay. Negative control predators were assayed for the presence of prey (rabbit IgG) in their gut by the ELISA described above. Mean (±SD) ELISA optical absorbance values were calculated for each predator. Individual predators that consumed a marked prey item were scored positive for protein remains if the optical absorbance value was three SDs above that of the negative control mean (Hagler *et al.*, 1992*b*).

Inclusion/exclusion field cages

Study site

Experiments were conducted at a 1.2-ha site at the USDA-ARS, Western Cotton Research Laboratory, Phoenix, AZ, USA. The field was planted with the commonly used full season cotton cultivar, 'Delta Pine 5415'. Many small areas in the field were left fallow to accommodate the transplanted cotton plants that were used for each experimental trial (see below). No insecticides were applied to the field.

Each trial was conducted on plants that were approximately the same size. Briefly, individual cottonseeds were planted every 2–3 weeks in 15.2-cm-diameter pots and grown in the greenhouse for ≈1 month as described by Hagler *et al.* (2004). One-month-old plants were transplanted in fallow portions of the field every 2–3 weeks. Water was continuously applied to the soil for 12 weeks using a drip irrigation system. The drip irrigation was turned off 1 week before each trial began. This method produced cotton plants that were 70.0 ± 15.6 cm tall with a surface area of 5359 ± 1709 cm^2.

Arthropod assemblage

The species composition, life stage and number of individuals released into each cage are listed in Table 1. This particular arthropod assemblage was selected because it contains a mix of arthropod herbivores, omnivores and carnivores commonly found in Arizona cotton fields (Hagler & Naranjo, 2005).

Predators

Adult coccinellids, *Hippodamia convergens* Guérin-Méneville and green lacewings, *Chrysoperla carnea* (Stephens) larvae were purchased from Nature's Control (Portland, OR, USA), a commercial supplier of beneficial organisms. The highly cannibalistic *C. carnea* larvae were kept in the individual rearing cells (e.g. a sheet of honeycomb Hexcell™ containing ≈600 larvae per sheet) that they were shipped in. The remaining members of the predator assemblage were collected from cotton and alfalfa fields located at the University of Arizona's Agricultural Research Station. All predator collections were made the day before each test. The collected predators were sorted by species in a light box at the laboratory and placed in separate Petri dishes and held overnight with water at 27°C.

Pests

The pests examined in this study included third instar *L. hesperus* and *T. ni* and *P. gossypiella* eggs. All pests were obtained from laboratory-reared colonies located at our facility. *L. hesperus* were reared on the artificial diet described by Patana & Debolt (1985), *T. ni* were reared on the artificial diet described by Chippendale & Beck (1965) and *P. gossypiella* were reared on the artificial diet described by Adkisson *et al.* (1960).

Pest-marking procedures

Lygus hesperus

A key ingredient of *L. hesperus* artificial diet is chicken egg white. Chicken egg white albumin can be readily detected using the anti-chicken IgG ELISA described by Hagler (1997). Newly hatched *L. hesperus* were fed artificial diet until they reached their third instar. An external mark was then applied to the third instar nymphs by spraying with 1.0 mL of a 5.0 mg mL^{-1} chicken IgG solution (Sigma Chemical Co., No. I-4881) using a medical nebuliser as described above. The nymphs were air-dried for 1 h before they were introduced into the cages (see below).

Trichoplusia ni

The larvae of *T. ni* were marked internally using the method described above for marking pink bollworm larvae. Briefly, newly hatched *T. ni* larvae were fed an artificial diet containing 0.5 mg of rabbit IgG per millilitre of diet until they reach their third instar. An external rabbit IgG mark was then applied to the third instar larvae by spraying with 1.0 mL of a 0.5 mg mL^{-1} rabbit IgG solution using a medical nebuliser as described above. The

Table 1 A listing of the arthropods, life stages and numbers released into each field cage (experimental unit). The 'ants included' treatment consisted of two trials exposed to four light treatments (eight cages total), and the 'ants excluded' cages consisted of a single trial exposed to four light treatments (four cages total).

			Cage Type	
Species	Life Stage	Classification[a]	Ants Included[b]	Ants Excluded[c]
Pectinophora gossypiella	Egg	Herbivorous pest	922/717 (6554)	719 (2877)
Trichoplusia ni	Third instar	Herbivorous pest	50/50 (400)	50 (200)
Lygus hesperus	Third instar	Omnivorous pest	50/50 (400)	50 (200)
Lygus hesperus	Adult	Omnivorous pest	30/10 (160)	50 (200)
Geocoris punctipes	Adult	Omnivorous predator	10/5 (59)	5 (19)
Spanagonicus albofasciatus	Adult	Omnivorous predator	10/0 (40)	0 (0)
Nabis alternatus	Adult	Omnivorous predator	10/4 (54)	4 (14)
Zelus renardii	Adult	Carnivorous	5/9 (56)	9 (36)
Collops vittatus	Adult	Carnivorous	20/10 (120)	10 (40)
Hippodamia convergens	Adult	Carnivorous	20/10 (120)	10 (40)
Chrysoperla carnea	Third instar	Carnivorous	140.5/10 (602)[d]	10 (40)
Misumenops celer (spider)	Adult	Carnivorous	5/4 (37)	4 (17)
Solenopsis xyloni	Adult	Carnivorous	NA[e]	NA[e]

[a]The primary feeding habit of each species.

[b]The number to the left of the slash is the number of individuals released in each of the four caged light treatments that included ants in the first trial; the number to the right of the slash is the number released in each of the four caged light treatments including ants during the second trial and the number in parenthesis is the cumulative total number of individuals released in the eight cages that included ants over both trials. In several instances, there were not enough field-collected predators captured to be released into each cage during the second trial. They are as follows: one cage only received four *G. punctipes*, no *S. albofasciatus* were collected (during the second trial), one cage only received two *N. alternatus* and one cage received four *M. celer*.

[c]The number of individuals released in each of the four caged light treatments that excluded ants in the second trial. The number in parenthesis is the cumulative total number of individuals released in the four cages that excluded ants. In several instances, there were not enough field-collected predators captured to be released into each cage during the second trial. They are as follows: one cage only received four *G. punctipes*, no *S. albofasciatus* were collected, one cage only received two *N. alternatus* and one cage received four *M. celer*.

[d]For the first trial, I averaged the number released (140.5) into each cage. I simply took the 600-cell sheet of Hexcell™ that the lacewings were shipped in, cut it into four equal sizes, counted the number of cells containing *C. carne*a and released the entire contents of one of the four sheets into each cage.

[e]Not applicable because the *S. xyloni* in the cages were from invading field populations.

larvae were air-dried 1 h before they were introduced into the cages (see below).

Pectinophora gossypiella

Pink bollworm eggs were obtained from egg sheets (Adkisson *et al.*, 1960) placed in our laboratory colony. The eggs did not require any protein mark because a pink-bollworm-egg-specific ELISA has been developed for this pest (Hagler *et al.*, 1994).

Experimental design

An experimental unit consisted of an individual cotton plant ≈0.70 m tall enclosed by a 1.0-m-long muslin sleeve cage. The bottom of each cage was tied at ground level around the base of the cotton plant with a permanent zip-tie (Thomas & Betts, Memphis, TN, USA). Each plant was vacuumed with a D-Vac for 2 min from the top opening of the cage to remove as many native arthropods as possible. Immediately after vacuuming, 10 square egg sheet patches (1.5 × 1.5 cm) containing ≈75–100 *P. gossypiella* eggs/patch were stapled to the underside of 10 randomly selected leaves. Then, third instar *T. ni* marked with rabbit IgG and third instar *L. hesperus* marked with chicken IgG were released into each cage (see Table 1 for release rates). Immediately after the pests were released, the top of each cage was tied with a wire twist tie to block movement of arthropods in and out of the cages. The mobile pests were given 1 h to disperse within each cage before the predaceous members of the assemblage were introduced into the cage. After 1 h, the remaining arthropods listed in Table 1 were introduced into each cage by quickly opening the top of the cage, releasing the predators and resealing the top of the cage with the wire twist tie. The insects were allowed to forage freely in each cage for 6 h. After 6 h, each cotton plant was cut at the base of the plant just below the zip-tie, placed in a large plastic garbage bag and frozen immediately at −70°C. Plant samples were processed by removing the plant from the freezer and painstakingly searching the entire cage for the remaining arthropods. Each arthropod was identified to species, counted and

ground (whole body) in 500 μL of TBS. Three separate 50 μL aliquots of each predator sample were used to assay for the presence of rabbit IgG (cabbage looper remains), chicken IgG (*Lygus* remains) and pink bollworm egg antigen by the three ELISAs described below.

Two experimental trials were conducted over a 24-h period on August 7–8 and September 25–26, 2003. Both trials contained photophase, scotophase, photo/scotophase and scoto/photophase cage treatments lasting 6 h. The photophase treatment was conducted under a continuous daylight phase from 0700–1300 h. The scotophase treatment was conducted under a continuous dark phase from 2200–0400 h. The photo/scotophase treatment was conducted under a 3-h light phase followed by a 3-h dark phase from 1800–2400 h. The scoto/photophase treatment was conducted under a 3-h dark phase followed by a 3-h light phase from 0200–0800. The first trial suffered from an unexpected invasion of native fire ants (*S. xyloni*) in the cages. Specifically, the fire ants invaded the muslin-covered plants through very small holes they chewed in the muslin (note: the holes were too small for the other arthropods to escape). Consequently, the second trial was modified to include both an ant and a no-ant treatment for each light cycle treatment. The cages excluding ants were surrounded by a 0.5-m-diameter garden liner. The bottom of the 15-cm-tall plastic garden liner was buried ≈5.0 cm under the ground, and the above-ground portion of the liner was heavily coated with Tanglefoot™ Spray on Formula Insect Trap Coating (Grand Rapids, MI, USA).

Data analysis

The percent mortality exhibited by each member of the assemblage was determined by dividing the number of each arthropod species found alive in each cage after the 6-h exposure interval by the number released into each cage. The results from the two cage trials that were invaded by ants were pooled together (note: there was only one ant-exclusion trial conducted for each of the four light phases). Descriptive charts were created to depict: (a) the overall percent mortality for each arthropod species and (b) the percent mortality of each targeted pest population that occurred during each light phase treatment.

Predator gut content evaluations

Protein-specific predator gut content ELISAs

Anti-rabbit IgG and anti-chicken IgG sandwich ELISAs were conducted on every recaptured predator to detect for the presence of prey (protein marks) in each predator's gut. The anti-rabbit ELISA is described above. The anti-chicken ELISA protocol is identical to the rabbit IgG ELISA except that the primary (Sigma Chemical Co., No. C-1161) and secondary antibodies (No. A9046) were anti-chicken IgGs developed in rabbit (see Hagler, 1997, for details).

Pink-bollworm-specific predator gut content ELISA

An indirect anti-*P. gossypiella*-egg-specific ELISA was also conducted on every recaptured predator to detect for the presence of pink bollworm egg antigen in their guts. This ELISA procedure and various characteristics of the ELISA (e.g. prey retention interval in various predator species, etc.) are described in detail by Hagler *et al.* (1994, 1997) and Hagler & Naranjo (1997).

Negative predator controls

Predators serving as negative controls (n = 8 per ELISA plate) were collected from nearby cotton and alfalfa fields. To ensure that these predators did not have any pink bollworm egg antigen in their gut prior to the analyses, they were isolated from any food source and only provided water for 3 days. Individual predators were then assayed by the three ELISAs described above. Mean (±SD) negative control optical density values were calculated for each species. An individual predator collected from each cage treatment was scored positive for each prey using the same criteria (e.g. mean + 3 SD) as described above.

Data analysis

The gut content ELISA results from all the cages (ants excluded and ants included) were pooled together. Descriptive charts were created to depict: (a) the overall percentage of each predator species positive for prey remains and (b) the percentage of each predator species positive for pest remains, with respect to each light cycle treatment.

Results

Laboratory feeding test

The results obtained from the earwig gut content analyses are given in Fig. 1A. The anti-rabbit IgG-specific ELISA was 100% effective at detecting a single rabbit-IgG-marked pink bollworm larva in the gut of an earwig for at least 48 h after feeding. The results obtained from the minute pirate bug gut content analyses are given

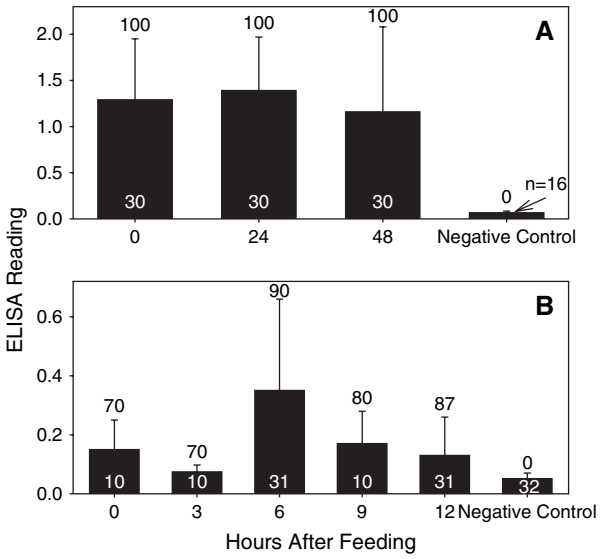

Figure 1 Mean (±standard deviation) ELISA readings for the retention of rabbit IgG in the gut of (A) an earwig that consumed a rabbit-IgG-marked fourth instar pink bollworm larva and (B) a minute pirate bug that consumed a rabbit-IgG-marked parasitoid (*Eretmocerus* sp.). The number above each error bar is the percentage of individuals positive for the presence of rabbit-IgG-marked prey. The number inside each bar is the number of individual predators tested for each treatment. The negative control predators consumed unmarked prey.

in Fig. 1B. This feeding trial showed that a single rabbit-IgG-marked parasitoid could be readily detected in the gut for at least 12 h after feeding.

Field cage test

Predator gut content analyses

A pink-bollworm-specific and two protein-specific ELISAs were conducted on each of the 1503 predators recaptured during the course of this study. The results from the predator gut content ELISAs were first pooled for each predator species, cage type and light cycle in order to simplify the data presentation and increase the sample sizes (i.e. the ant exclusion cage portion of the study had small sample sizes). The gut content ELISAs revealed that spiders [19.2%, primarily *Misumenops celer* (Hentz)], *Nabis alternatus* Parshley (17.1%), *Zelus renardii* (10.7%) and *Collops vittatus* (8.3%) had the highest proportion of individuals containing marked *L. hesperus* nymphs; *C. vittatus* (22.5%), *Spanagonicus albofasciatus* (Reuter) (20.0%) and *G. punctipes* (8.2%) had the highest proportion of individuals containing pink bollworm egg antigen and spiders (21.2%), *S. albofasciatus* (20.0%) and *N. alternatus* (11.4%) had the highest proportion of individuals containing rabbit-IgG-marked cabbage looper larvae (Fig. 2, red cross-hatched bars). Interestingly, none of the few ($n = 29$) surviving *C. carnea* larvae yielded a positive ELISA reaction for the presence of any of the targeted prey. The data were then pooled for each predator species and cage type to show the impact that the light cycle had on predation. Overall, the most frequent predation events occurred during the photo/scotophase and photophase light cycles (Fig. 2).

Inclusion/exclusion field cage test

The degree that the predator assemblage fed on the targeted pests (e.g. interguild predation) is presented in Fig. 3A. The results indicate that the percent mortality of each pest population was high, particularly in those cages that included ants. This indicates that ants and other unidentified predators in the assemblage are major mortality factors on this pest assemblage.

The degree of intraguild predation occurring among the predator assemblage is given in Fig. 3B. The most striking indication that intraguild predation might be occurring among the assemblage was evidenced by the 96.2 and 85.0% mortality of the *C. carnea* larval population in the cages that included and excluded ants, respectively (Fig. 3B). Other predator species in this study that also suffered relatively high mortality rates in the cages containing ants included adult *S. albofasciatus* (62.5%), *N. alternatus* (53.7%) and *C. vittatus* (25.8%). The percent mortality of predators in cages that excluded ants was much lower ranging from 0% for *H. convergens* to 30% for *N. alternatus* (Fig. 3B).

The percent mortality of the pests during the four light cycle treatments is given in Fig. 4. The percent mortality for *L. hesperus* nymphs was lowest (32%) during the photophase and about the same (52–54%) during the other light phases in the cages that were invaded by ants (Fig. 4A). The percent mortality of *L. hesperus* nymphs in the cages excluding ants ranged from 12% during the scoto/photophase to 28% during the scotophase (Fig. 4B). The percent mortality for *P. gossypiella* eggs was high throughout the day and regardless of whether ants were present or not. However, the percent mortality was always highest during the photophase and lowest during the scotophase (Fig. 4C and Fig. 4D). The percent mortality for *T. ni* larvae was highest during the photophase and scoto/photophase light cycles in both the cage treatments, with the overwhelming majority of predation occurring in the cages containing ants (Fig. 4E and Fig. 4F).

There were only a few noticeable patterns of intraguild predation occurring in the ant inclusion and exclusion cages with respect to the light cycle that the predator assemblage was exposed to (data not shown). For the ant inclusion cages, the percent population reduction of *C. carnea* was always >92%, regardless of the light cycle.

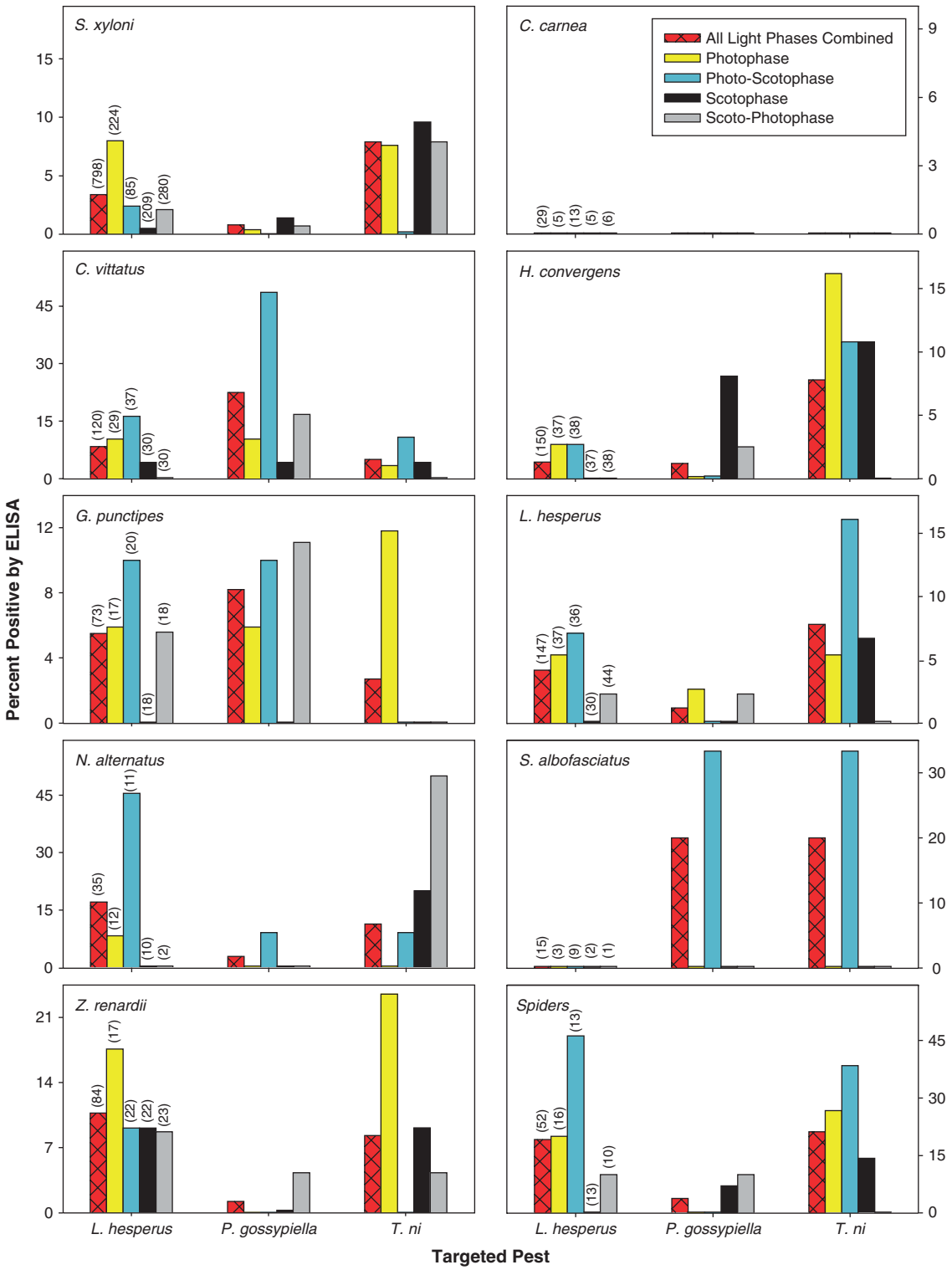

Figure 2 The percentage of predators positive by each ELISA (both cage types combined) for the presence of the three targeted pests during the four light cycle treatments. The numbers in parenthesis above the bars are the number of individual predators assayed by each ELISA for the photophase, photo/scotophase, scotophase and scoto/photophase light cycles, respectively. The red cross-hatched bars represent the percentage of positive ELISA reactions for all four light phases combined.

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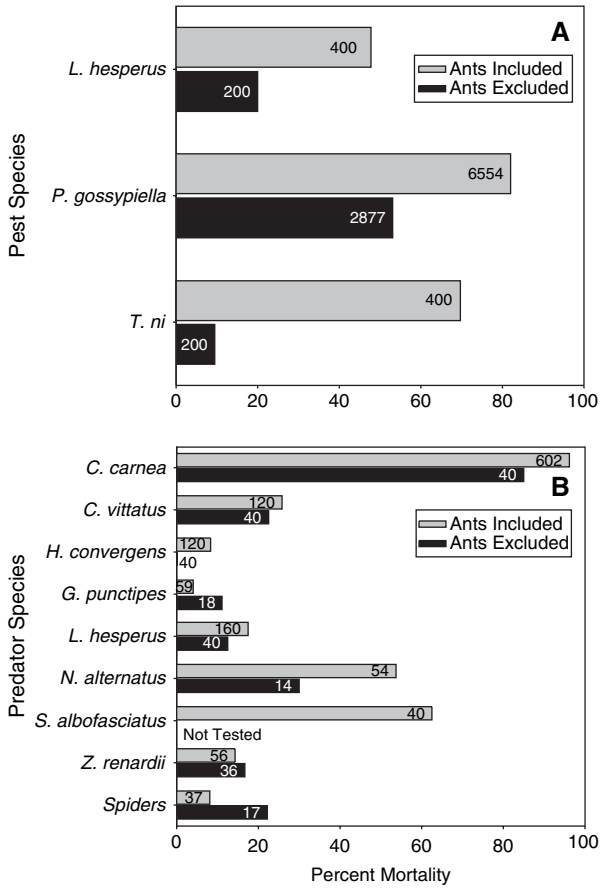

Figure 3 (A) The overall (all light phases combined for each cage type) percent mortality of the pest populations after 6-h exposure to the arthropod assemblage that either included foraging ants (grey bars) or excluded ants (black bars). (B) The overall (all light phases combined for each cage type) percent mortality of the predator populations after 6-h exposure to the arthropod assemblage that either included foraging ants (grey bars) or excluded ants (black bars). The numbers in each shaded bar represent the total number of individual pests released into the cages.

Over half of the total mortality for *C. vittatus* (52%) occurred during the scotophase light cycle. For the ant exclusion cages, *C. carnea* (≥80%) was readily devoured during each light cycle, almost all predation on *C. vittatus* (89%) occurred during the photophase, and all predation on *N. alternatus* occurred during the photo/scotophase (data not shown).

Discussion

Laboratory feeding test

The predators and prey selected for the laboratory feeding tests were chosen because they represent extreme-case scenarios for detecting predation using molecular gut content assays. The easy-case scenario was a striped earwig, a large chewing predator, feeding on a relatively large protein-marked pink bollworm larva. The 100% positive ELISA results yielded by the earwigs for up to 24 h after eating a marked pink bollworm larva prompted the investigation of a 'tough-case' predator gut content ELISA consisting of a minute pirate bug, a small piercing and sucking predator, feeding on a very small protein-marked parasitoid. The majority of minute pirate bugs scored positive for the presence of rabbit-IgG-marked parasitoids up to 12 h after feeding. The data yielded from the laboratory feeding tests suggest that a wide variety of insect prey can be marked with a unique protein, and the subsequent detection of the marked prey can be detected in the guts of a wide variety of predators by a protein-specific ELISA.

Field cage test

The primary goal of this research was to develop and validate an alternative method for precisely and simultaneously identifying predation on several targeted species inhabiting a relatively complex arthropod assemblage. Rosenheim *et al.* (1995) stated that there is a critical need for methods to study the population ecology of multispecies interactions simultaneously. The method described here combines previous research using a pest-specific predator gut content ELISA developed to detect pink bollworm egg predation (Hagler *et al.*, 1994) with protein-marking ELISAs developed to detect protein marks put on arthropods for dispersal studies (Hagler *et al.*, 2002; Blackmer *et al.*, 2004; Hagler & Naranjo, 2004). Previously, ELISAs using either pest-specific polyclonal (PAbs) or monoclonal antibodies (MAbs) have been used to identify key predators of single pests (Ragsdale *et al.*, 1981; Hagler *et al.*, 1992*b*, Bacher *et al.*, 1999; Symondson *et al.*, 1999; Harwood *et al.*, 2004). The simplicity and low cost of ELISA lends itself to the efficient analysis of hundreds of field-collected predators per day (Hagler & Naranjo, 2005). However, PAbs are often not species specific, and insect-specific MAb development is too technically difficult, costly and time consuming for studies investigating the prey choices of predators occupying a complex arthropod assemblage (Greenstone & Shufran, 2003). PCR assays using pest-specific DNA probes might be less expensive to develop than MAb-based ELISAs (Greenstone & Shufran, 2003), but a PCR assay is more costly, complex, tedious and time consuming than an ELISA (Sheppard & Harwood, 2005; de Leon *et al.*, 2006). Due to these limitations, only a few studies have been conducted where more than one prey species in an assemblage has been targeted for predator gut content analysis (Hagler & Naranjo, 1994*a*,*b*; Agustí *et al.*,

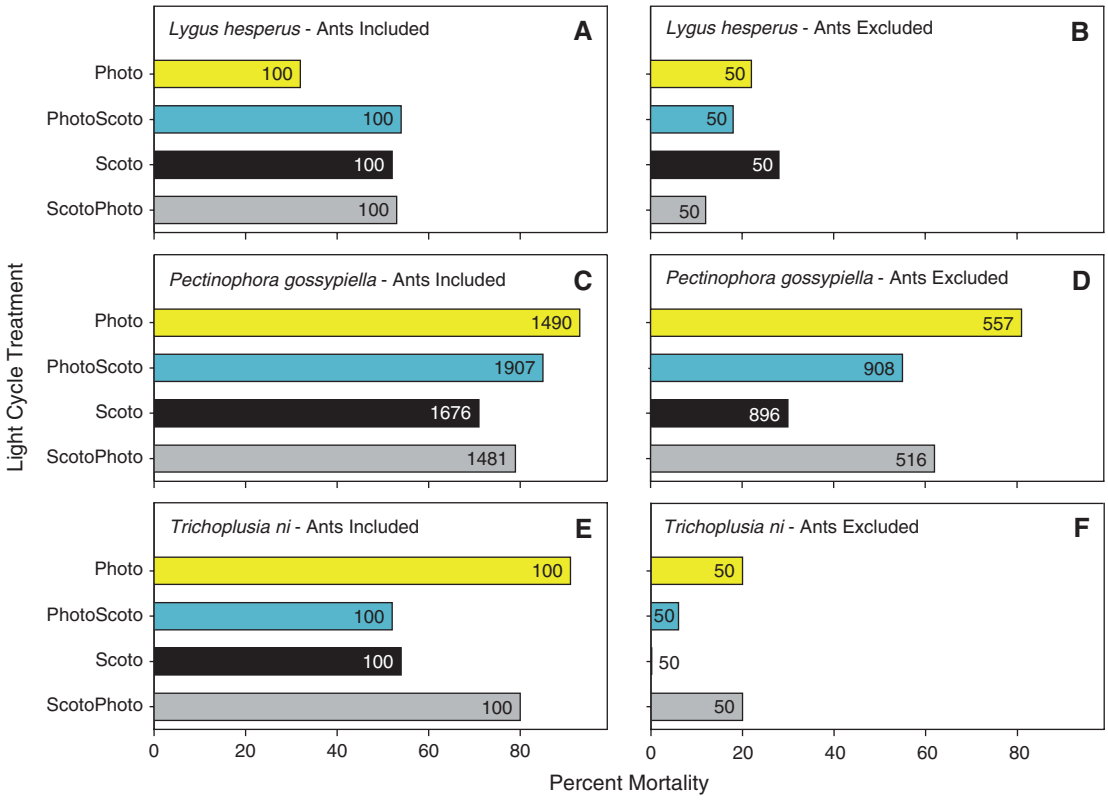

Figure 4 The percent mortality of the targeted pest populations during each light cycle after 6-h exposure to the arthropod assemblage that either included foraging ants or excluded ants. The numbers in each shaded bar represent the number of individual pests released into the cages.

2003; Kasper *et al.*, 2004; Harper *et al.*, 2005) and even fewer studies have included a large sample size of predators (Hagler & Naranjo, 1994*a*,*b*; Kasper *et al.*, 2004).

The results from the laboratory feeding trials described here show that exogenous protein placed on prey can be readily detected in the guts of predators that consumed a single marked prey item (Fig. 1). This technique when combined with field cage techniques could expand the use of molecular gut content assays for precisely and simultaneously deciphering complex predator–prey interactions.

The cage inclusion/exclusion portion of this study showed that ants were a major source of mortality to the arthropod assemblage (Figs 3 and 4). However, the gut content analyses revealed that only 0.8%, 3.4% and 7.9% of the ants assayed contained *P. gossypiella*, marked *L. hesperus* and marked *T. ni*, respectively (Fig. 2). The relatively low frequency of positive ELISA responses yielded by the ants might be best explained by Nuessly & Sterling (1986). They observed that *S. invicta* foraging on *Heliothis zea* (Boddie) eggs marked with ^{32}P usually captured their prey intact (e.g. these ants rarely scored positive for the presence of ^{32}P) and then rapidly returned to the nest. Once at the nest, the ^{32}P-marked eggs were disseminated by the foraging ants to their nest mates.

Another objective of this study was to use the multiple gut content ELISAs to determine if certain predators feed preferentially during specific times of the day. Pfannenstiel & Yeargan (2002) determined by direct field observation that *Nabis* spp. and phalangiid spiders prey almost exclusively at night on lepidopteran eggs, while other predators tend to be more active (but not exclusive) during the day or night. Previous predator gut content evaluations identified *C. vittatus* and *L. hesperus* as major predators and *N. alternatus* as a minor predator of the pink bollworm egg stage. The predators examined in these previous studies were collected from the field between 0600 and 1000 h throughout the duration of the experiments (Hagler & Naranjo, 1994*a*,*b*). Subsequent research showed that the detection of pink bollworm egg antigen in the guts of predators varied greatly between species (Hagler & Naranjo, 1997; Hagler *et al.*, 1997). For example, the pink bollworm detection half-life of the indirect ELISA ranged from <1.0 h for *H. convergens* to 13.5 h for *Orius insidiosus*. The results obtained by Pfannenstiel & Yeargan (2002) clearly indicate that future gut content

analyses should be conducted on predators collected throughout the day in order to eliminate any potential bias that may be associated with predator circadian feeding activity. The results yielded from this study showed that the majority of predation occurred during the photo/scotophase and photophase light cycle treatments.

The overall results (e.g. the data yielded from each light phase was combined for each cage type) from the cage inclusion/exclusion portion of this study concur with the results of others which show that generalist predators do not discriminate between herbivores, omnivores or carnivores (Rosenheim *et al.*, 1993; Polis, 1994; Cisneros & Rosenheim, 1997; Eubanks & Denno, 2000) and suggest that fire ants are voracious generalist predators (Fig. 3). These results support the findings of previous studies that used similar inclusion/exclusion cage methodology. For example, Rosenheim *et al.* (1993) evaluated the degree of intraguild predation occurring in a relatively simple cotton arthropod assemblage containing aphids (*Aphis gossypii* Glover) and lacewings (*C. carnea*) together or aphids, lacewings plus *Z. renardii*, *G. punctipes* and *Nabis* spp. They showed that *C. carnea* alone was effective at suppressing aphid populations. However, lacewing populations decreased and the aphid populations increased when the other generalist predators (especially *Z. renardii* and *Nabis* spp.) were selectively added to the assemblage. They concluded that the higher order predators fed preferentially on lacewings, and in turn, hindered the biological control of aphids by lacewings. Rosenheim *et al.* (1993) duly noted, however, that the inclusion/exclusion cage methodology they used only provided a crude estimate of the degree of intraguild predation occurring. This was because of the fact that field cage methods alone cannot precisely and simultaneously delineate which members of a complex assemblage are engaging in interguild or intraguild predation.

Limitations of the technique

Protein-specific gut content ELISAs can be a powerful method for identifying complex food web interactions. However, I would be remiss if I did not discuss the potential limitations of this technique. First, ELISA false positives as a result of third trophic level interactions can lead to the misidentification of the actual predator that consumed a marked prey item (Harwood *et al.*, 2001). This is a drawback also shared with other molecular gut content analyses (e.g. pest-specific ELISA, PCR, electrophoresis and elemental marking) (Sheppard *et al.*, 2005). Second, protein marks might transfer to other insects by nonpredatory events such as direct contact. In a previous study, individual male *P. gossypiella* moths, marked with rabbit IgG, were placed in small rearing cups with individual unmarked virgin females. After two days of close contact (including mating), the ELISA results revealed that 100% of the marked males and only 4% of the unmarked females contained rabbit IgG (Hagler & Miller, 2002). Further studies are needed to ensure that external marks are not passed from marked prey to predators during nonpredatory events. However, the low frequency of lateral transfer of rabbit IgG between scaly moths in very confined arenas suggest that there is only a slight chance that protein marks will transfer from marked to unmarked arthropods by direct contact in relatively large field cages. Third, some predators are cannibalistic (e.g. *C. carnea* and *L. hesperus*). Cannibalism cannot be accounted for because the devoured arthropod would be eliminated from the arena, but the same protein mark would be passed to its conspecific. This potential drawback actually identifies another potential use of this technique. Specifically, each individual of a single targeted species (instead of different species) can be marked with a specific protein. In turn, every predator in the assemblage can be assayed by a multitude of protein-specific ELISAs to quantify predation on the targeted species. Quantification of predation is the major limitation of all the current post-mortem gut contents assays described above. Currently, studies are underway designed to quantify predation by an assemblage on individually marked *C. carnea*. Fourth, scavenging predators could have a profound effect on the interpretation of the ELISA results. It has been determined that carrion prey can be easily detected by pest-specific ELISA and PCR assays (Sunderland, 1996; Calder *et al.*, 2005; Foltan *et al.*, 2005; Juen & Traugott, 2005). There is no doubt that protein markers would also transfer to predators feeding on protein-marked carrion prey. Again, this technique can be used to decipher this complex interaction. Finally, field cages are an essential component to this prey-marking procedure. Unfortunately, field cage experiments do not exactly replicate what occurs in nature because they are generally short-term studies and the cages can affect attack rates, immigration, etc. (Luck *et al.*, 1988).

In short, multiple protein markers and protein-specific gut content ELISAs used in concert with field cage methodology can provide researchers with a simple, precise, rapid, economical and sensitive method for studying a wide variety of predator–prey interactions. In this study, I demonstrated that the technique can be used to track the predation events of individual predators on three targeted pest insect species within a complex arthropod assemblage without the burden of developing a pest-specific ELISA or PCR assay for each of the targeted species. This technique can be further expanded to

qualify or quantify the degree of: (a) interguild and intraguild predation, (b) hunting versus scavenging predation and (c) cannibalism occurring in arthropod assemblages.

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Note

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